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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

Attorney's Docket No. 2577-109

U.S. Application No. (if known, see 37 CFR 1.5) 09/857841

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/SG98/00103	11 December 1998	11 December 1998

TITLE OF INVENTION ENHANCED PROTEIN PRODUCTION IN HIGHER PLANTS BY N-TERMINAL FUSION OF A UBIQUITIN OR A CUCUMBER MOSAIC VIRUS COAT PROTEIN PEPTIDE

APPLICANT(S) FOR DO/EO/US: Rong-Xiang FANG, Jun-Lin WU, and Xiao-Ying CHEN.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has **NOT** expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

ITEMS 11. TO 16. below concern other document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A **FIRST** preliminary amendment.
    [ ] A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information: Courtesy copy of the published International Application.

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U.S. APPLICATION NO. (If known, see 37 CFR 1.50) 09/857841	INTERNATIONAL APPLICATION NO. PCT/SG98/00103	ATTORNEY DOCKET NO. 2577-109

				CALCULATIONS	PTO USE ONLY
17. [X] The following fees are submitted: **Basic National Fee (37 CFR 1.492)(a)(1)-(5):** Search Report has been prepared by the EPO or JPO $ 860.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) $ 690.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) $ 710.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $ 1,000.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $ 100.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				$ 1,000.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$ 130.00	
Claims	Number Filed	Number Extra	Rate		
Total Claims	3 2 -20 =	12	X $18.00	$ 216.00	
Independent Claims	13 - 3 =	10	X $80.00	$ 800.00	
Multiple dependent claim(s) (if applicable)			+ $270.00	$	
TOTAL OF ABOVE CALCULATIONS =				$2,146.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$2,146.00	
Processing fee of $130.00 for furnishing the English translation later [ ] 20 [ ] 30 than months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$2,146.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$2,146.00	
				Amount to be refunded	$
				charged	$

a. [X] A check in the amount of <u>$2,146.00</u> to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. 02-2135 in the amount of $_________ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2135. A duplicate copy of this sheet is enclosed.

d. [ ] Payment by credit card. (Form PTO-2038 enclosed.)

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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TITLE OF THE INVENTION

ENHANCED PROTEIN PRODUCTION IN HIGHER PLANTS BY N-TERMINAL FUSION OF A UBIQUITIN OR A CUCUMBER MOSAIC VIRUS COAT PROTEIN PEPTIDE

BACKGROUND OF THE INVENTION

Strategies for production of proteins in heterologous fusion form have been widely applied in biotechnology for many purposes, such as secretion of proteins from host cells (fused to signal peptides), easy detection or purification of protein products (fused to reporter enzymes for detection and to peptide tags for purification), searching for proteins with desired biological activities (e.g., in the phage display technique and the two-hybrid system). Enhanced expression of proteins of interest has also been achieved by N-terminal fusion of a small peptide to the target protein. Fusion of a ubiquitin gene together with a ubiquitin promoter to the 5'-end of a gene of interest is one of the systems which has been used to enhance protein expression. Ubiquitin exists in all eukaryotic cells and is the most highly conserved protein yet identified. It is abundant in cells and exhibits profound stability to heat and proteolytic degradation. Moreover, ubiquitin precursors, that is, polyubiquitin where ubiquitin monomers are linked up head to tail and ubiquitin extension proteins where a single ubiquitin is appended at its C-terminus to either of two small ribosomal proteins, undergo rapid processing by ubiquitin C-terminal hydrolases, which cleave C-terminal of the ubiquitin moieties and release the free ubiquitin monomer and the C-terminal extension proteins. All of these features have rendered ubiquitin as an excellent N-terminal fusion partner to augment target protein accumulation in genetic engineering.

The ubiquitin fusion approach was first developed by Butt et al. (1989), who showed that fusion of ubiquitin to yeast metallothionein or to the α subunit of the adenoylate cyclase-stimulatory GTP-binding protein increased the yield of these otherwise unstable or poorly expressed proteins from undetectable levels to 20% of the total cellular proteins in *E. coli*. Ecker et al. (1989) demonstrated that in yeast, ubiquitin fusion resulted in enhanced expression of three mammalian proteins by up to 200-fold and all these ubiquitin fusion proteins were correctly processed by yeast ubiquitin-specific endopeptidase to release authentic functional proteins. A similar yeast ubiquitin fusion expression system was reported by Sabin et al. (1989), in which ubiquitin/human γ-interferon and ubiquitin/α1-proteinase inhibitor were highly expressed and quantitatively cleaved to yield γ-IFN and α1-PI with authentic amino termini.

Since these early reports, a wealth of studies on ubiquitin fusion expression of various proteins in *E. coli* and yeast have been described (Baker et al., 1994; Barr et al., 1991; Coggan et al., 1995; Gali and Board, 1995; Gehring et al., 1995; Han et al., 1994; Kiefer et al., 1992; Lu et al., 1990; Lyttle et al., 1992; Mak et al., 1989; McDonnell et al., 1989; McDonnell et al., 1991; Pilon et al., 1996; Poletti et al., 1992; Rian et al., 1993; Tan and Board, 1996; Welch et al., 1995). Very often fusion to ubiquitin led to dramatic enhancement in yield of the fusion protein in bacteria, or of the cleaved product in yeast.

Enhanced expression of foreign proteins by ubiquitin fusion has also been observed in plants. In analysis of the promoter of the tobacco polyubiquitin gene, *Ubi.U4*, by driving transient expression of the GUS reporter in tobacco protoplasts, Genschik et al. (1994) found deletion of the intron sequence from the *Ubi.U4* fragment spanning from -263 to the end of the first ubiquitin-coding unit had no detectable influence on the GUS activity, but further deletion of the ubiquitin-coding sequence diminished the GUS activity by 55%.

None of these studies has shown the direct enhancing function of the ubiquitin fusion from a heterologous promoter. Garbarino and Belknap (1994) observed that fusion of the promoter plus ubiquitin-coding region of the potato ubiquitin extension protein gene *ubi 3* to the GUS reporter gene resulted in GUS activity 5- to 10-fold higher than the direct fusion of the *ubi 3* promoter to the GUS gene did in transgenic potato. Again, the synergistic effect of the *ubi 3* promoter and the ubiquitin-coding sequence on the enhanced GUS activity was not excluded. In another study with a potato polyubiquitin gene, *ubi 7*, the same group (Garbarino et al., 1995) demonstrated that in transgenic potato plants GUS expression level from the fusion construct containing the *ubi 7* promoter-5' untranslated sequence-intron-first ubiquitin coding unit was 10 times higher than that derived by only the *ubi 7* promoter with the 5' untranslated sequence. However, the effects of the intron and the ubiquitin protein fusion in increasing expression level of the GUS reporter were not clearly discriminated.

In addition to the above mentioned journal papers, a number of patents related to the ubiquitin fusion technology have been filed since 1989. They are shown in Table 1. The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference, and for convenience are respectively grouped in the appended List of References.

Table I
Patents related to the ubiquitin fusion technology

Title	Inventor	Patent No.	Filing Date	Host cells
Generating desired amino-terminal residue in protein	MIT	WO 8909829	10/19/1989	
Regulation metabolic stability of a protein	MIT	US 5093242	3/3/1992	mammal, yeast
Nucleic acid constructs, malaria polypeptides and vaccines	Chiron	WO 9208795	5/29/1992	yeast
Production of a protein with a predetermined amino-terminal amino acid residue	MIT	US 5196321	3/23/1993	E. coli
Yeast expression system for retinoid-X receptor	American Cyanamid	EP 608532	8/3/1994	yeast
Recombinant DNA vectors	Mascarenhas	WO 9423040	10/13/1994	E. coli
New heat-inducible N-degron protein and nucleic acid encoding it	Varshavsky, Dohmen, Johnston, Wu	WO 9521269	8/10/1995	
Fusion proteins containing the N-or C-terminal of ubiquitin	Varshavsky, Johnston	WO 9529195	11/2/1995	
New fusion protein of ubiquitin plant and lytic peptide	Carbarino, Jaynes, Belknap	WO 9603519	2/8/1996	plant
Production of tissue factor pathway-inhibitor in yeast cells	Innis, Creasey	WO 9604377	2/15/1996	yeast
Stable recombinant ubiquitin-lytic peptide fusion protein	J. Jaynes	WO 9603522	2/8/1996	plant
Fusion protein encoded by a gene construct	Bachmair, Finley, Varshavsky	US 5496721	5/3/1990	mammal, yeast

SUMMARY OF THE INVENTION

In accordance with the present invention a method for enhancing expression of proteins in plants or plant cells is achieved by the fusion of a ubiquitin monomer coding sequence to the 5' end of the coding sequence of the proteins. Expression of the ubiquitin fusion proteins is driven by a promoter other than promoters from polyubiquitin protein genes or ubiquitin extension protein genes. Thus enhancement of expression level of the proteins is due to the 5' terminal addition of the ubiquitin monomer coding sequence. The ubiquitin fusion proteins are cleaved at the carboxy-terminal glycine 76 residue of the ubiquitin, presumably by plant ubiquitin specific proteases, to produce proteins with desired biological properties. A second aspect of this invention is that the N-terminal peptide of 14 amino acid residues of cucumber mosaic virus coat protein (NP14) can be used as an N-terminal fusion partner to increase the expression level of target proteins in plants. The N-terminal fusion approaches described in this invention allow higher yield production of proteins in plants, either in the authentic forms in the ubiquitin fusion system or as the fusion protein in the NP14 fusion system.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleotide sequence and deduced amino acid sequence of tobacco *ubi.NC89*. The nucleotide sequence is listed as SEQ ID NO:1 and the amino acid sequence is SEQ ID NO:2 in the Sequence Listing. The primers used in PCR are underlined and the mended 37-mer oligonucleotide is double-underlined.

Figure 2 shows the synthetic DNA coding for the 14 N-terminal amino acids of CMV CP (NP14). The nucleotide sequence is SEQ ID NO:3 and the amino acid sequence is SEQ ID NO:4.

Figure 3 illustrates the construction of the ubiquitin-GUS fusion protein expression vector pUG. The nucleotide sequence shown for pSKUBC1 is SEQ ID NO:5, the sequence shown for pBI221 is SEQ ID NO:6, and the sequence shown for pUG is SEQ ID NO:7.

Figure 4 illustrates the construction of the NP14-GUS fusion protein expression vector pCG. The nucleotide sequence shown for pUCG2 is SEQ ID NO:8.

Figure 5 illustrates the construction of the ubiquitin-luciferase fusion protein expression vector pUL. The arrow marked in the recognition sequence of Stu I in pBIubi indicates the end of the ubiquitin coding region and the cleavage site of the ubiquitin fusion protein. The upper

nucleotide sequence shown for pBIubi is SEQ ID NO:9, the lower nucleotide sequence shown for pBIubi is SEQ ID NO:10, and the nucleotide sequence shown for pUL is SEQ ID NO:11.

Figure 6 illustrates the construction of the NP14-luciferase fusion protein expression vector. The nucleotide sequence shown for pCL is SEQ ID NO:12.

Figure 7 illustrates the construction of ubiquitin-GUS fusion/LUC dual report binary vector pUGL121.

Figure 8 illustrates the construction of the NP14-GUS fusion/LUC dual reporter binary vector pCGL121.

Figure 9 illustrates the construction of the GUS/LUC dual reporter binary vector pBIL121.

Figure 10 illustrates the ubiquitin fusion cloning vector pBIubi. The upper nucleotide sequence is SEQ ID NO:13 and the lower nucleotide sequence is SEQ ID NO:14.

Figure 11 illustrates the NP14 fusion cloning vector pBINP14.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to methods and constructs for enhancing protein production in plants. The methods comprise fusing an expression-enhancing nucleic acid at the 5' terminus of the gene for which enhanced expression is desired. In one aspect of the invention, a ubiquitin gene is inserted in front of the gene encoding the desired protein such that a fusion protein is produced wherein ubiquitin is directly fused to the amino terminus of the desired protein. Enzymes such as C-terminal hydrolases, will cleave at the C-terminus of the ubiquitin in the fusion protein thereby releasing the desired protein in its natural form as well as forming free ubiquitin. The presence of the ubiquitin gene in the resulting fusion protein results in enhanced expression of the gene thereby yielding a greater amount of the desired protein product than occurs in the absence of the ubiquitin gene. It is necessary to use only the coding portion of the ubiquitin gene. The ubiquitin promoter is unnecessary, and the ubiquitin gene fusion can be under the control of a heterologous promoter.

In a second aspect of the invention, enhanced protein production is seen when a nucleic acid encoding 14 amino acids of cucumber mosaic virus coat protein is placed in front of the gene encoding a desired protein such that a fusion protein is produced wherein the fusion protein

includes the 14 amino acids of the cucumber mosaic virus coat protein at the amino terminus of the fusion protein.

The aspects of the invention are set out in the following Examples which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized. Techniques such as transfection of protoplasts, preparation of transgenic tobacco plants, fluorometric GUS assays and luciferase assays are well known to those of skill in the art and are not described in detail herein.

EXAMPLE 1

DNA Sequences Coding for the Tobacco Ubiquitin and the N-terminal Peptide of CMV Coat Protein

The coding sequence of the ubiquitin monomer contains 228 base pairs. The 5' part of 191 base pairs was obtained by polymerase chain reaction (PCR) amplification on the total DNA of *Nicotiana tobacum* var. NC89 and the remaining 37 base pairs were prepared as a synthetic oligonucleotide. An SphI site encompassing the initiation codon ATG and an NcoI site following the last codon GGC were created to facilitate cloning. The tobacco ubiquitin coding sequence was then cloned into pGEM-5ZF and sequenced. Figure 1 shows the DNA sequence and the deduced amino acid sequence of the tobacco ubiquitin. The 76-amino acid sequence is identical to that derived from a tobacco polyubiquitin gene *ubi.U4* (Genschik et al., 1994). However, the nucleotide sequence of the region amplified from the tobacco DNA is different from the corresponding regions of all ubiquitin monomers found in *ubi.U4*. We have named this tobacco ubiquitin coding sequence as *ubi.NC89*.

The cucumber mosaic virus coat protein (CMV CP) is encoded by the viral subgenomic RNA 4 and comprises 218 amino acid residues. The CP gene of the strain CMV-SD was cloned by RT-PCR (Guo et al., 1993) and the cDNA sequence encoding the 14 N-terminal amino acids (NP14) was either cut out of the CP gene by NcoI/AccI digestion or chemically synthesized. In the synthesized version of the NP14 coding sequence (Figure 2), overhanging adapters for BamHI and SstI sites were attached to the 5'- and 3'-ends, respectively, for easy cloning.

EXAMPLE 2

Translational Fusion Constructs for Transient Expression Assays

A. Ubiquitin-GUS fusion construct pUG

The *ubi.NC89* sequence was taken from the plasmid pSKUBC1 as an XbaI-NcoI (filled-in) fragment and inserted into the XbaI-BamHI (filled-in) site upstream of the GUS gene in pBI221 to construct pUG as shown in Figure 3.

B. NP14-GUS fusion construct pCG

Plasmid pUCG2 is a derivative of pBI221, in which the *ubi.NC89* sequence and the NP14 sequence, linked as a read-through ORF, was inserted into the XbaI-SmaI sites in front of the GUS gene. The ubiquitin moiety was removed from pUCG2 by XbaI-SacII digestion and pCG was formed by recircularizing. Figure 4 illustrates these steps clearly.

C. Ubiquitin-LUC fusion construct pUL

An NcoI (filled-in)-SstI fragment containing the firefly luciferase (LUC) gene was inserted into the ubiquitin fusion vector pBIubi (see Figure 10) downstream of *ubi.NC89* via the StuI-SstI sites in the polylinker region, resulting in pUL as shown in Figure 5.

D. NP14-LUC fusion construct pCL

The NcoI (filled-in)-SstI fragment containing the LUC gene was inserted into the NP14 fusion vector pBINP14 (see Figure 11) downstream of the NP14 coding sequence via AccI (or SalI which is the equivalent site here) (filled-in)-SstI sites, resulting in pCL as shown in Figure 6.

EXAMPLE 3

GUS/LUC Dual Reporter Constructs for Stable Transformation

To examine the enhancing effects of the N-terminal addition of the ubiquitin or CMV CP NP14 on GUS expression in stably transformed plants, a series of GUS/LUC (test/reference) dual reporter constructs were made. Essentially they are based on the fusion constructs used in transient expression assays, namely, pUG and pCG. The chimeric GUS expression cassettes were moved into the plant transformation intermediate plasmid pBI121, resulting in pUG121 and

pCG121, respectively. The expression cassette of the reference reporter LUC, which was constructed by replacing the GUS gene in pBI221 with the LUC gene, was pre-made as a HindIII fragment (HindIII-35S/LUC/NOS-HindIII) and then inserted into the unique HindIII site of pUG121, pCG121 and pBI121, respectively. The resulting GUS/LUC dual reporter constructs, pUGL121, pCGL121 and pBIL121 are shown in Figures 7, 8 and 9, respectively.

EXAMPLE 4

Ubiquitin fusion enhances the expression of GUS and LUC in tobacco protoplasts

The ubiquitin-GUS fusion construct pUG or the control plasmid pBI221 was introduced into tobacco protoplasts derived from tobacco BY-2 suspension cells, together with a reference plasmid FFO which contained LUC gene driven by the 35S promoter. GUS activities were determined and normalized by luciferase activities. In four independent transfection experiments, the normalized GUS activities (ΔGUS) from pUG were considerably higher than those from pBI221. The averaged increase fold due to the ubiquitin fusion is 6.0 (Table 2). When using LUC as a reporter and GUS as an internal standard as expressed from pBI221, the normalized LUC activities from pUL were 1.37 to 3.11 fold higher than those from the control plasmid p35SLUC (35S-LUC-NOS) in three independent transfection experiments, with the average increase fold about 2 (Table 3).

EXAMPLE 5

CMV CP NP14 Is a More Efficient Fusion Partner than Ubiquitin

The enhancing effects of the NP14 fusion on GUS and LUC expression in tobacco protoplasts were examined in experiments parallel to the above mentioned ubiquitin fusion study. The NP 14-GUS fusion construct pCG produced an average 11-fold higher GUS activity than did pBI221. These results are shown in Table 2. Fusion of NP14 to LUC increased the LUC activity by 2.87 times, calculated by comparing the normalized LUC activity of pCL to that of p35SLUC. These results are shown in Table 3. It is apparent that NP14 is a more efficient fusion partner than ubiquitin in augmenting GUS and LUC expression in tobacco cells.

Table 2

Normalized GUS activities and enhancing fold of the N-terminal fusion constructs

plasmid	pBI221	pUG		pCG	
activities	GUS	ΔGUS	E	ΔGUS	E
1	293.3	3760.0	12.8	5743.0	19.6
2	206.7	584.3	2.8	940.8	4.6
3	856.7	3733.8	4.4	6708.0	7.8
4	100.0	408.8	4.1	1247.0	12.5
average E value		6.0±2.2		11.1±3.2	

Notes: 1. The normalized GUS activity ΔGUS is calculated by the formula

$$\Delta GUS_n = \frac{GUS_n \times LUC_{221}}{LUC_n}$$

where n represents a particular GUS fusion construct, 221 represents pBI221.

2. The enhancing fold E is calculated as $\frac{\Delta GUS_n}{GUS_{221}}$

Table 3

Normalized LUC activities and enhancing fold of the N-terminal fusion constructs

Plasmid		p35S LUC		pUL			pCL		
activities		ΔLUC	average ΔLUC	ΔLUC	average ΔLUC	E	ΔLUC	average ΔLUC	E
1	1	252	290	274	396	1.37	457	491	1.70
	2	329		518			529		
2	1	169	169	556	526	3.11	701	794	4.70
	2	ND		496			886		
3	1	64	112	141	164	1.46	270	246	2.20
	2	160		181			254		
	3	ND		170			214		
Mean±SE				1.98±0.56			2.87±0.92		

Notes: 1. The normalized LUC activity ΔLUC is calculated by the formula

$$\Delta LUC_n = \frac{LUC_n \times GUSp35SLUC}{GUS_n}$$

where n represents a particular LUC fusion construct.

2. The enhancing fold E is calculated as

$$\frac{\Delta LUC_n}{LUCp35SLUC}.$$

EXAMPLE 6

Ubiquitin- and NP14-fusion Enhance GUS Expression in Transgenic Plants

To examine the enhancing effects of the ubiquitin fusion and the NP14 fusion on GUS expression in stably transformed plants, three GUS/LUC (test/reference) dual reporter constructs were made based on the binary vector pBI121. pUGL121, pCGL121 and pBIL121 contained expression cassettes ubiquitin-GUS, NP14-GUS and GUS only (control), respectively, and the reference LUC expression cassette was integrated in each plasmid (Figures 7-9). Tobacco plants transformed with each of the three constructs were prepared and analyzed for GUS and LUC activities. Each plant was analyzed twice in two independent experiments and only those plants displaying reasonable consistency of the relative GUS activities (GUS/LUC) in two experiments were included for comparison. As shown in Table 4, although variations in the relative GUS activities existed among different transformants from the same constructs, the average GUS expression level of 5 qualified plants containing the 35S-ubiquitin/GUS fusion construct was 4 times higher than that derived from 6 plants containing the 35S-GUS construct, confirming the enhancing effect of the ubiquitin fusion on GUS expression as previously observed in tobacco protoplasts. Again, the NP14 fusion displayed a higher enhancing effect on GUS expression than did the ubiquitin fusion. The average relative GUS activity of 14 pCGL plants was about 7 fold that derived from the pBIL121 construct.

EXAMPLE 7

Ubiquitin fusion and NP14 fusion cloning vectors

pBIubi (Figure 10) and pBINP14 (Figure 11) are two fusion protein expression vectors allowing for insertion of target genes downstream of the *ubi.NC89* and the CMV CP NP14 coding sequence, respectively. Both vectors are derivatives of pBI221, with the GUS gene being replaced by the *ubi.NC89* or NP14 coding sequence. In pBIubi, a polylinker sequence was attached to the 3' end of the *ubi.NC89* sequence and the penultimate codon of the *ubi.NC89* was changed from GGT to GGA for creating a StuI site in the polylinker region. In pBINP14, two cloning sites, SalI (here equivalent to an AccI site) and SstI, are available for cloning the target genes downstream from the NP14 sequence (the last 5 base pairs of the NP14 sequence form part of the SalI recognition sequence). In order to use AccI instead of SalI for cleaving pBINP14, the AccI site at -393 of the CaMV 35S promoter was eliminated.

Table 4

Effects of ubiquitin- and NP14-fusion on GUS expression in transgenic tobacco plants

Plant lines	Relative GUS activities: GUS/LUC (pmol MU·min^{-1}/cpm x 10^{-3})								
	pUGL121			pCGL121			pBIL121		
	exp. 1	exp. 2	average	exp. 1	exp. 2	average	exp. 1	exp. 2	average
1	12.9	15.3	14.1	2.4	3.4	2.9	1.4	2.6	2
2	13	43	28	4.5	6.8	5.65	5.2	2.4	3.8
3	0.7	0.5	0.6	63.2	9.5	36.35	4.2	0.6	2.4
4	0.3	0.4	0.35	26.9	8.3	17.6	2.5	5.4	3.95
5	4.8	0.8	2.8	17.8	22.2	20	0.4	0.38	0.39
6				2.1	5	3.55	0.5	0.82	0.66
7				4.6	5.8	5.2			
8				58.7	20.2	39.45			
9				15.6	3.6	9.6			
10				17.2	4.4	10.8			
11				3	1.4	2.2			
12				17.9	24.2	21.05			
13				20.7	19.4	20.05			
14				13.7	25.3	19.5			
Mean ±SE	9.17±5.34			15.28±3.18			2.2±0.61		

While the invention has been disclosed by reference to the details of preferred embodiments of the invention, it is to be understood that the disclosure is intended in an illustrative rather than in a limiting sense, as it is contemplated that modifications will readily occur to those skilled in the art, within the spirit of the invention and the scope of the appended claims.

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PCT Publication No. WO 89/09829

PCT Publication No. WO 92/08795

PCT Publication No. WO 94/23040

PCT Publication No. WO 95/21269

PCT Publication No. WO 95/29195

PCT Publication No. WO 96/03519

PCT Publication No. WO 96/04377

PCT Publication No. WO 96/03522

EP 608532

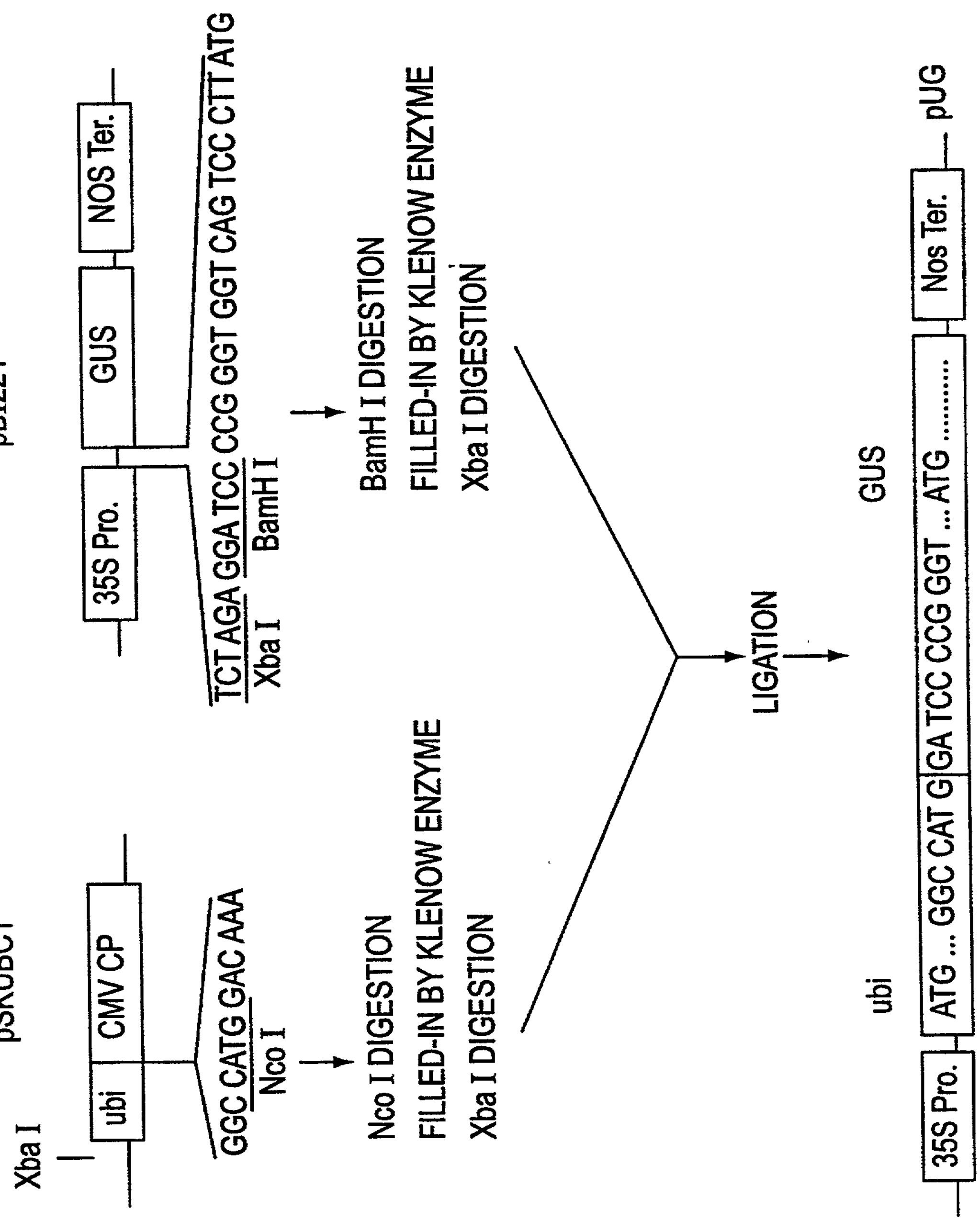
```
SphI
GC ATG CAG ATC TTC GTA AAG ACC CTG ACG GGG
 1  M   Q   I   F   V   K   T   L   T   G
   AAG ACT ATT ACC TTA GAG GTA GAG TCA TCG
11  K   T   I   T   L   E   V   E   S   S
   GAC ACC ATT GAC AAT GTT AAG GCT AAG ATT
21  D   T   I   D   N   V   K   A   K   I
   CAG GAC AAG GAA GGC ATT CCA CCG GAC CAG
31  Q   D   K   E   G   I   P   P   D   Q
   CAG CGG TTG ATT TTC GCA GGT AAG CAG CTT
41  Q   R   L   I   F   A   G   K   Q   L
   GAG GAT GGC CGA ACA CTA GCT GAC TAC AAC
51  E   D   G   R   T   L   A   D   Y   N
   ATC CAG AAG GAG TCC ACT CTC CAT CTC GTC
61  I   Q   K   E   S   T   L   H   L   V
   TTA AGA CTC CGC GGT GGC CATGG
71  L   R   L   R   G   G
                          NcoI
```

FIG. 1

```
BamHI
   1  M    D   K    S   E    S   T   S
 GATCCATG GAC AAA TCT GAA TCA ACC AGT
      TAC CTG TTT AGA CTT AGT TGG TCA

 A    G   R    N   R    R   14
GCT GGT CGT AAC CGT CGA CGAGCT
CGA CCA GCA TTG GCA GCT GC
                 AccI    SstI
```

FIG. 2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Xba I
ubi
CMV CP
GGC CATG GAC AAA
Nco I
Nco I DIGESTION
FILLED-IN BY KLENOW ENZYME
Xba I DIGESTION
pBI221
35S Pro.
GUS
NOS Ter.
TCT AGA GGA TCC CCG GGT GGT CAG TCC CTT ATG
Xba I
BamH I
BamH I DIGESTION
FILLED-IN BY KLENOW ENZYME
Xba I DIGESTION
LIGATION
ubi
GUS
35S Pro.
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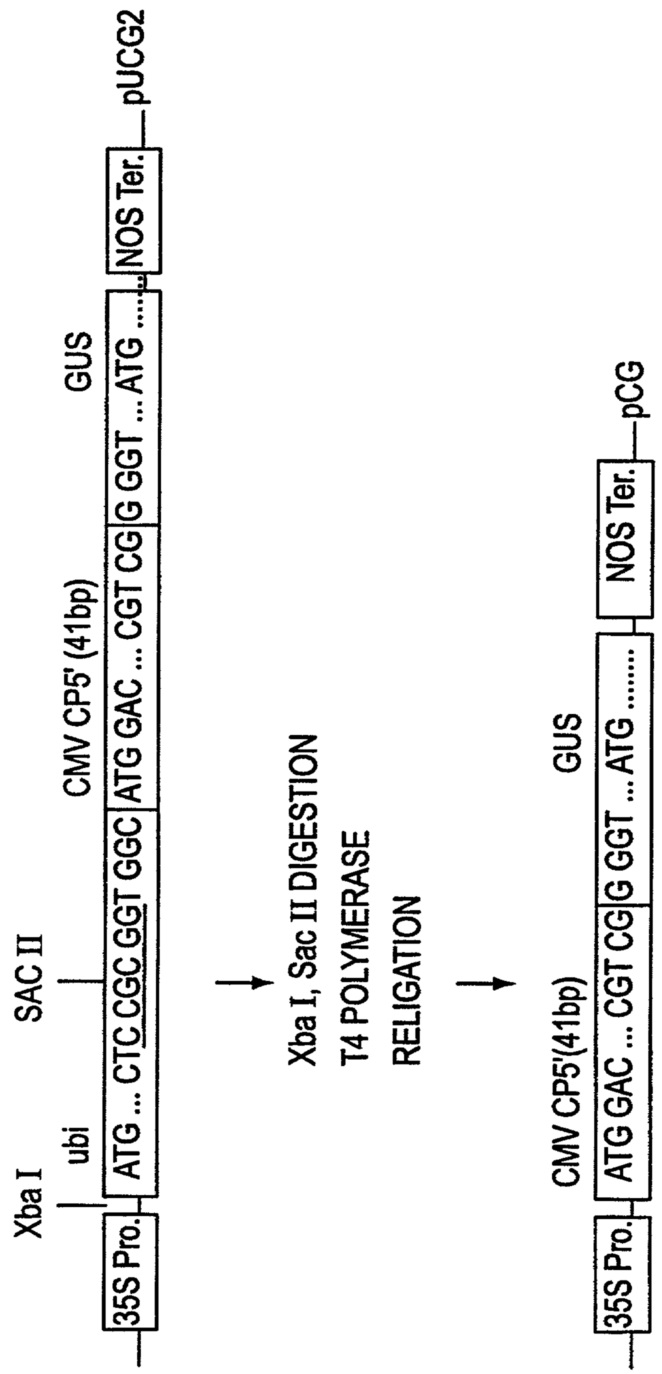
Nos Ter.
pUG

FIG. 3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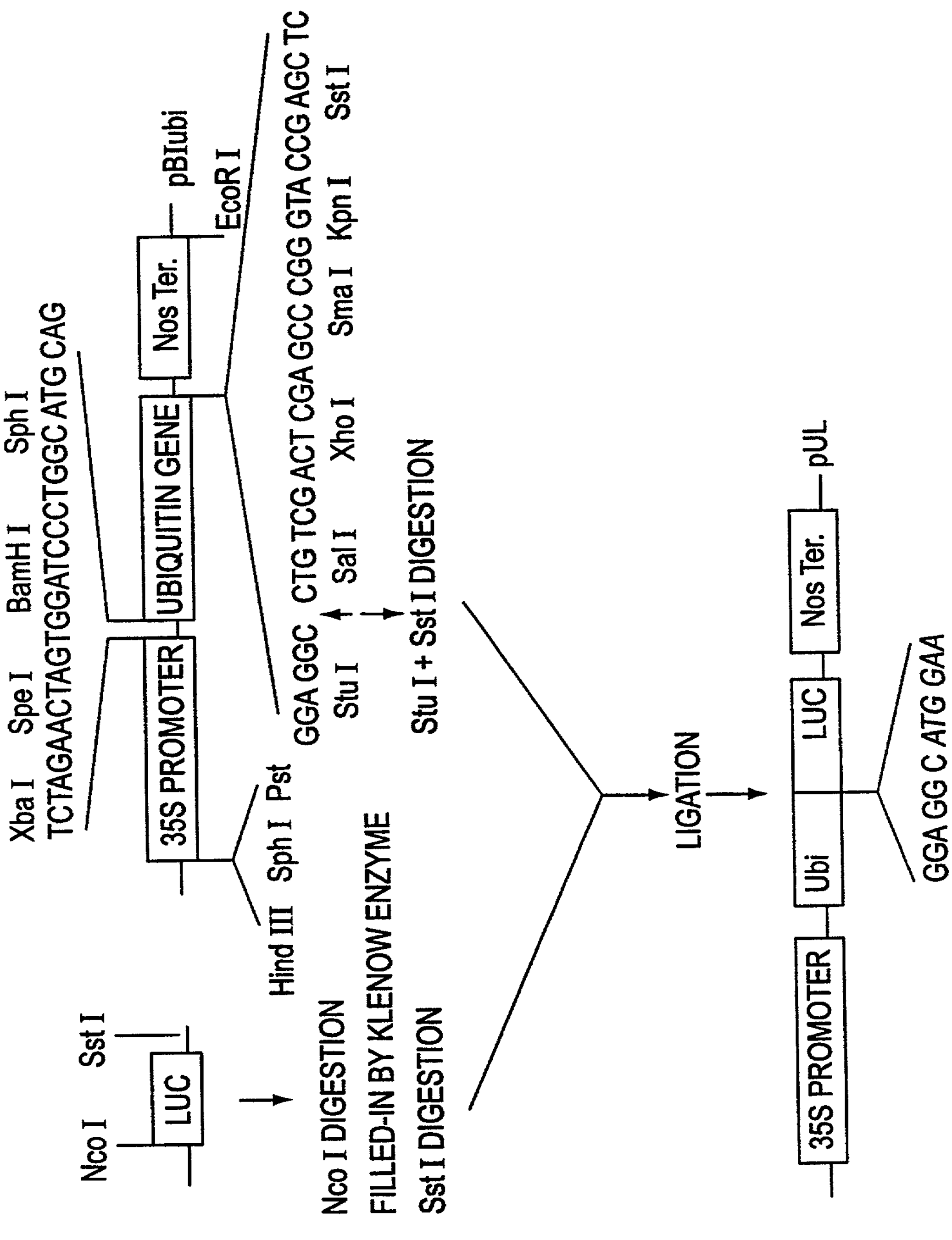
ubi
SAC II
35S Pro.
ATG ... CTC CGC GGT GGC
CMV CP5' (41bp)
ATG GAC ... CGT CG
G GGT ... ATG ....
GUS
NOS Ter.
pUCG2
Xba I, Sac II DIGESTION
T4 POLYMERASE
RELIGATION
35S Pro.
CMV CP5'(41bp)
ATG GAC ... CGT CG
G GGT ... ATG ....
GUS
NOS Ter.
pCG

FIG. 4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Spe I
BamH I
Sph I
TCTAGAACTAGTGGATCCCTGGC ATG CAG
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UBIQUITIN GENE
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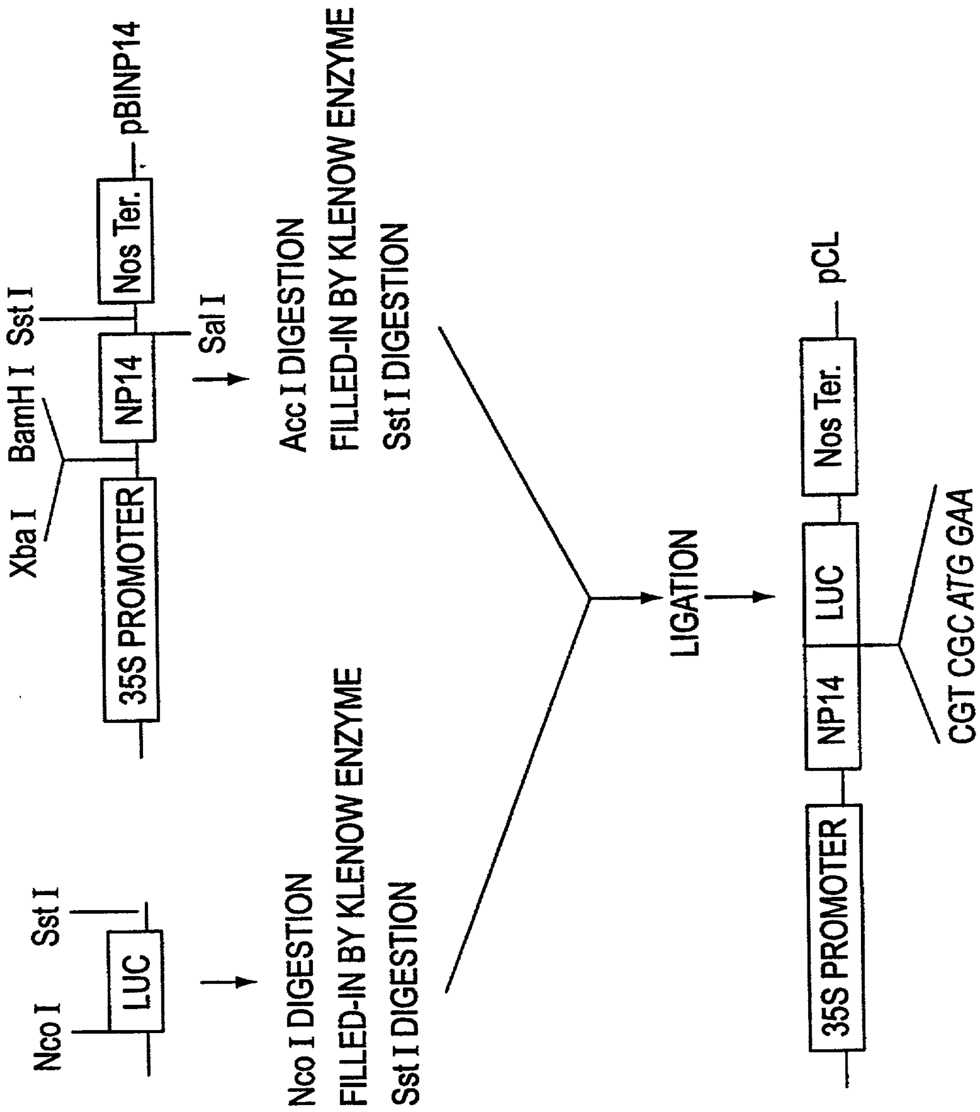
pBIubi
EcoR I
Hind III
Sph I
Pst
GGA GGC
CTG TCG ACT CGA GCC CGG GTA CCG AGC TC
Stu I
Sal I
Xho I
Sma I
Kpn I
Sst I
Stu I + Sst I DIGESTION
Nco I
Sst I
LUC
Nco I DIGESTION
FILLED-IN BY KLENOW ENZYME
Sst I DIGESTION
LIGATION
35S PROMOTER
Ubi
LUC
Nos Ter.
pUL
GGA GG C ATG GAA

FIG. 5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Sst I
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NP14
Nos Ter.
pBINP14
Sal I
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FILLED-IN BY KLENOW ENZYME
Sst I DIGESTION
Nco I
Sst I
LUC
Nco I DIGESTION
FILLED-IN BY KLENOW ENZYME
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LIGATION
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Nos Ter.
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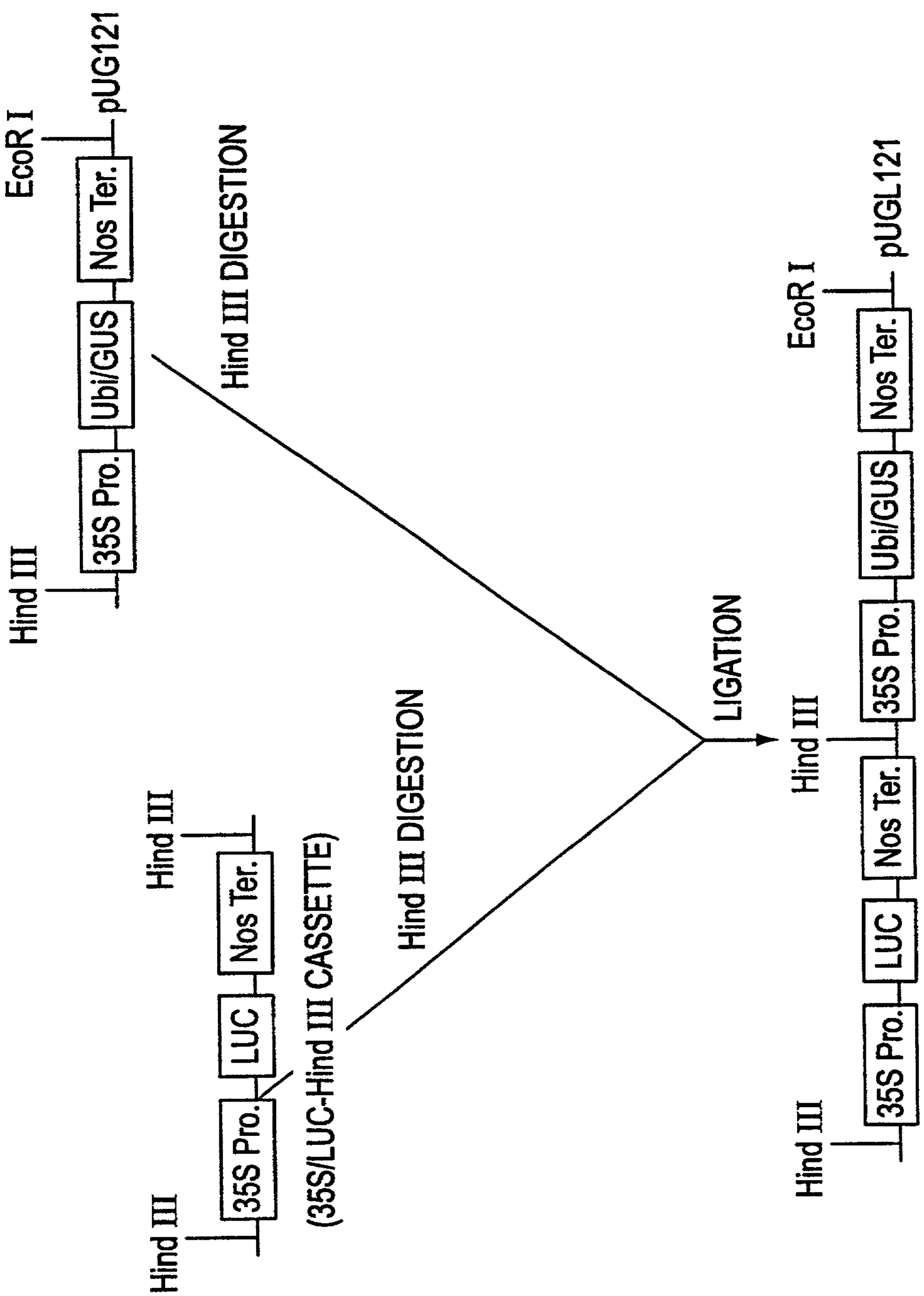
FIG. 6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Ubi/GUS
Nos Ter.
EcoR I
pUG121
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Hind III
35S Pro.
LUC
Nos Ter.
Hind III
(35S/LUC-Hind III CASSETTE)
Hind III DIGESTION
LIGATION
Hind III
35S Pro.
LUC
Nos Ter.
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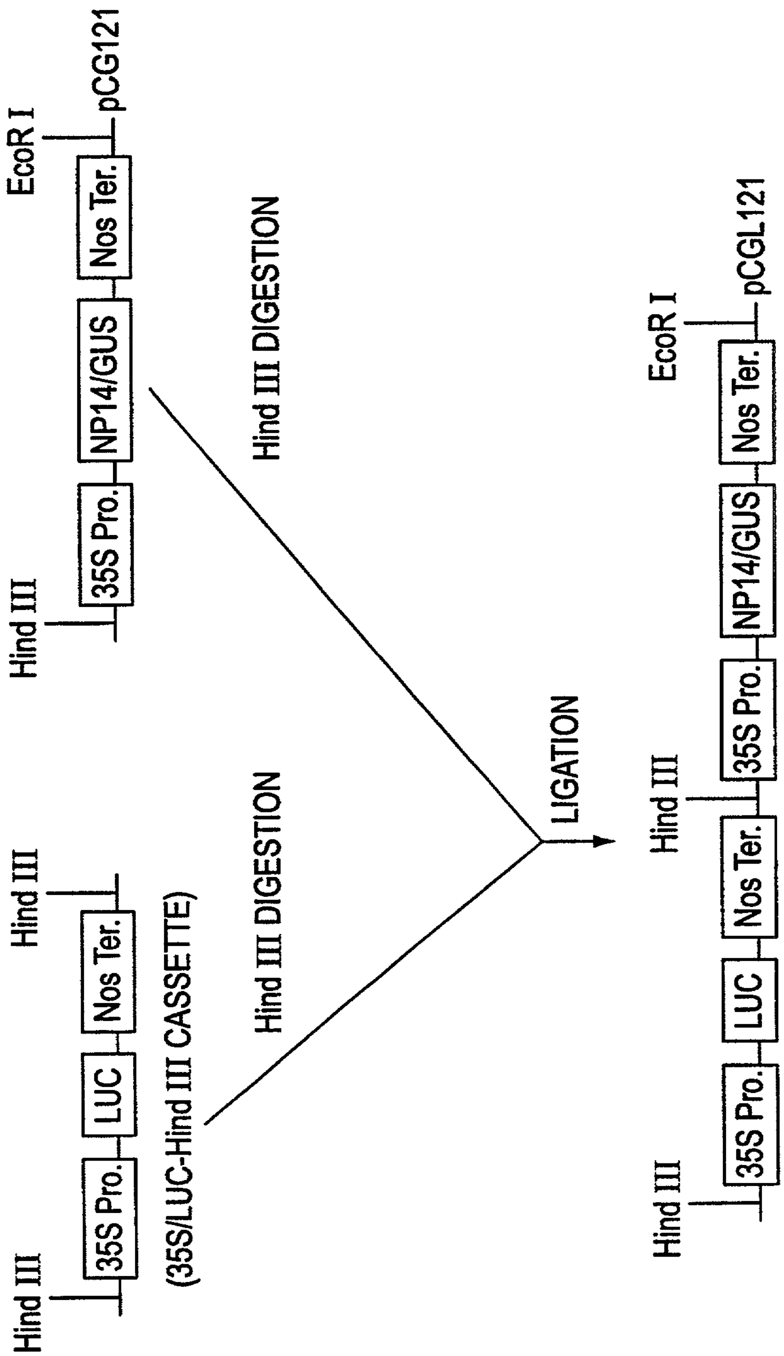
Ubi/GUS
Nos Ter.
EcoR I
pUGL121

FIG. 7



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NP14/GUS
Nos Ter.
EcoR I
pCG121
Hind III DIGESTION
Hind III
35S Pro.
LUC
Nos Ter.
Hind III
(35S/LUC-Hind III CASSETTE)
Hind III DIGESTION
LIGATION
Hind III
35S Pro.
LUC
Nos Ter.
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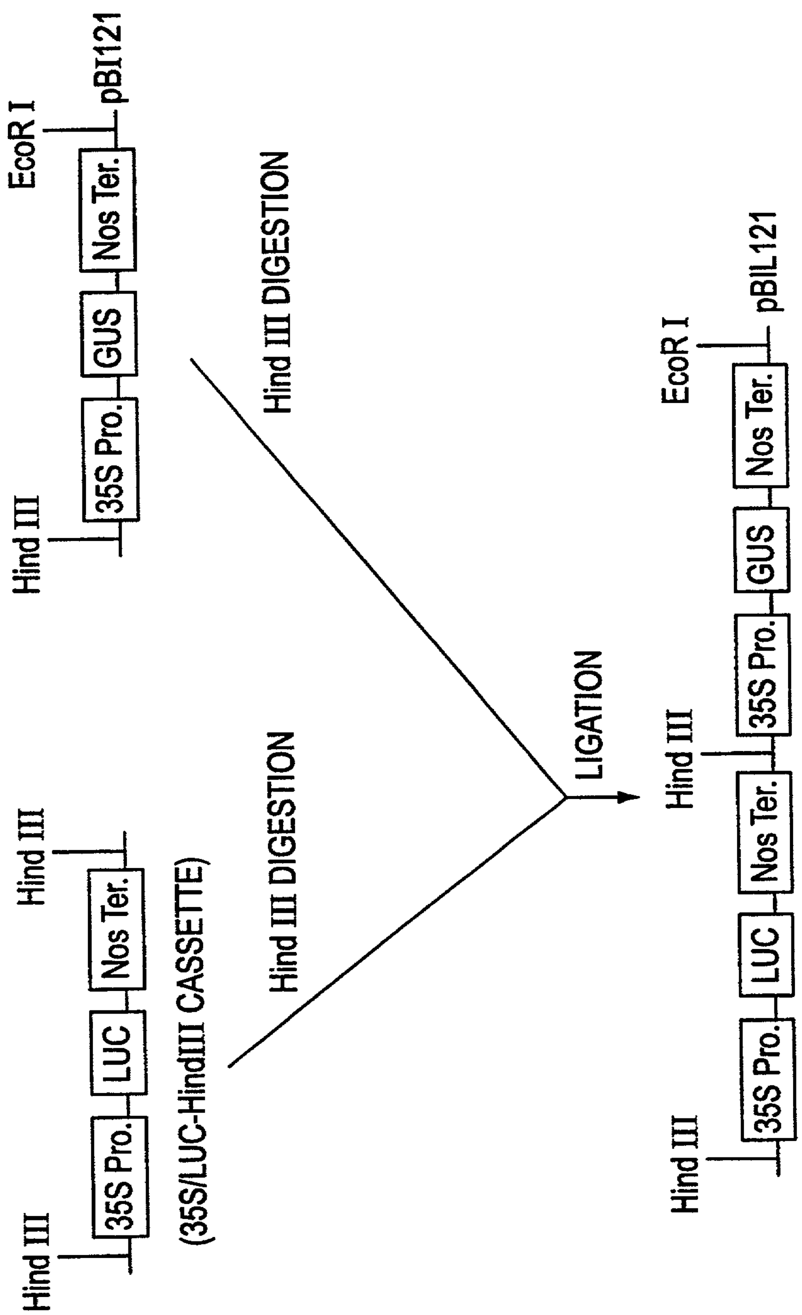
Nos Ter.
EcoR I
pCGL121

FIG. 8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LUC
Nos Ter.
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Hind III
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GUS
Nos Ter.
EcoR I
pBI121
Hind III DIGESTION
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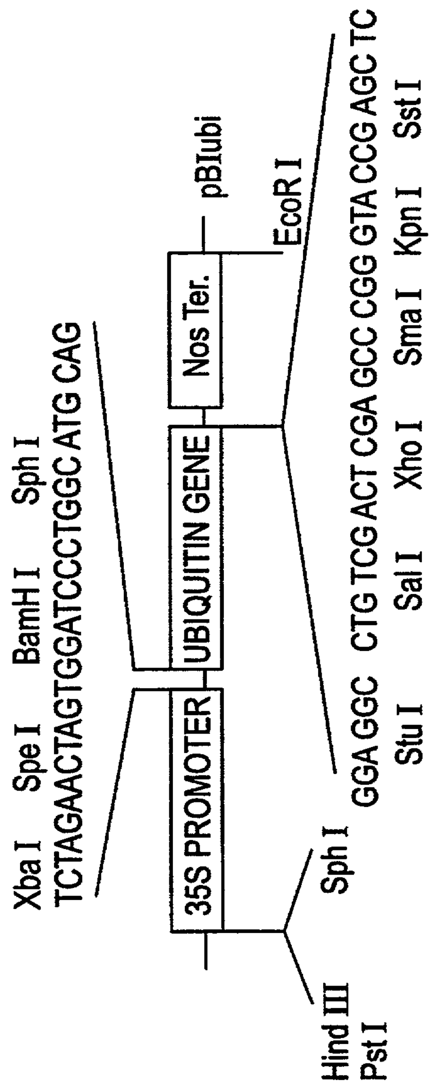
FIG. 9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Spe I
BamH I
Sph I
TCTAGAACTAGTGGATCCCCTGGC ATG CAG
35S PROMOTER
UBIQUITIN GENE
Nos Ter.
pBIubi
EcoR I
Hind III
Pst I
Sph I
GGA GGC CTG TCG ACT CGA GCC CGG GTA CCG AGC TC
Stu I
Sal I
Xho I
Sma I
Kpn I
Sst I

FIG. 10

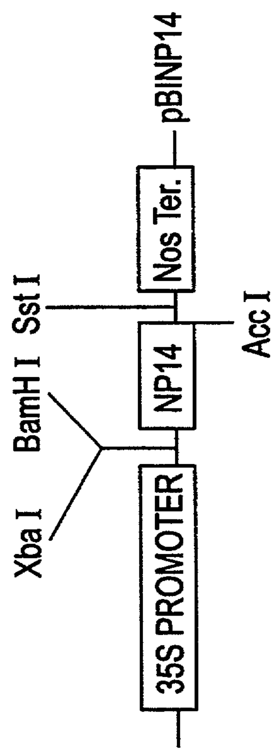

FIG. 11

# DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)	Attorney Docket No.	2577-109
	First Named Inventor	Rong-Xiang FANG
	COMPLETE IF KNOWN	
	Application Number	09/857,841
☐ Declaration Submitted with Initial Filing	Filing Date	December 11, 1998
☒ Declaration Submitted after Initial Filing	Group Art Unit	To Be Inserted
	Examiner Name	Not Yet Assigned

As a below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: ENHANCED PROTEIN PRODUCTION IN HIGHER PLANTS BY N-TERMINAL FUSION OF A UBIQUITIN OR A CUCUMBER MOSAIC VIRUS COAT PROTEIN PEPTIDE the specification of which was filed on December 11, 1998 as PCT International Application Number PCT/SG98/00103.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Numbers	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			☐ ☐	☐ ☐	☐ ☐

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

I or we hereby appoint the registered practitioner(s) associated with Customer No. **6449** to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. Direct all correspondence to Customer Number **6449**.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF SOLE OR FIRST INVENTOR:	[ ] A petition has been filed for this unsigned inventor		
Given Name: Rong-Xiang (first and middle [if any])		Family Name: FANG or Surname	
Inventor's Signature R. X. Fang		Date 22 AUG 2001	
Residence: City: Beijing	State	Country: China CNX	Citizenship: China
Mailing Address: Building 944, Apt. 203, Zhong Guan Cun Nanlu			
Mailing Address			
City: Beijing	State	Zip: 100086	Country: China
NAME OF SECOND INVENTOR:	[ ] A petition has been filed for this unsigned inventor		
Given Name: Jun-Lin (first and middle [if any])		Family Name: WU or Surname	
Inventor's Signature Wu Jun-Lin		Date 22 AUG 2001	
Residence: City: McLean	State: VA VA	Country: U.S.	Citizenship: China
Mailing Address: 1919 Kennedy Drive, #102			
Mailing Address			
City: McLean	State: VA	Zip: 22102	Country: U.S.
NAME OF THIRD INVENTOR:	[ ] A petition has been filed for this unsigned inventor		
Given Name: Xiao-Ying (first and middle [if any])		Family Name: CHEN or Surname	
Inventor's Signature Chen Xiaoying		Date 22 AUG 2001	
Residence: City: Beijing	State	Country: China CNX	Citizenship: China
Mailing Address: Kexueyuannanli, Building 303, Apt. 304, Datun Road, Chaoyang District			
Mailing Address			
City: Beijing	State	Zip: 100101	Country: China
NAME OF FOURTH INVENTOR:	[ ] A petition has been filed for this unsigned inventor		
Given Name (first and middle [if any])		Family Name or Surname	
Inventor's Signature		Date	
Residence: City	State	Country	Citizenship
Mailing Address			
Mailing Address			
City	State	Zip	Country

SEQUENCE LISTING

<110> Fang, Rong-Xiang

<120> ENHANCED PROTEIN PRODUCTION IN HIGHER PLANTS BY N-TERMINAL FUSION OF A UBIQUITIN OR A CUCUMBER MOSAIC VIRUS COAT PROTEIN PEPTIDE

<130> 2248-109

<140>
<141>

<160> 14

<170> PatentIn Ver. 2.0

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<213> Nicotiana tabacum

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<220>
<223> Modified from wild-type to insert an SphI site in the region encompassing the initiation codon ATG and to insert an NcoI site following the last codon GGC.

<400> 1

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     1               5                  10                  15

gag gta gag tca tcg gac acc att gac aat gtt aag gct aag att cag     95
Glu Val Glu Ser Ser Asp Thr Ile Asp Asn Val Lys Ala Lys Ile Gln
                20                  25                  30

gac aag gaa ggc att cca ccg gac cag cag cgg ttg att ttc gca ggt    143
Asp Lys Glu Gly Ile Pro Pro Asp Gln Gln Arg Leu Ile Phe Ala Gly
            35                  40                  45

aag cag ctt gag gat ggc cga aca cta gct gac tac aac atc cag aag    191
Lys Gln Leu Glu Asp Gly Arg Thr Leu Ala Asp Tyr Asn Ile Gln Lys
        50                  55                  60

gag tcc act ctc cat ctc gtc tta aga ctc cgc ggt ggc catgg          235
Glu Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly
    65                  70                  75
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1 5 10 15

Val Glu Ser Ser Asp Thr Ile Asp Asn Val Lys Ala Lys Ile Gln Asp
20 25 30

Lys Glu Gly Ile Pro Pro Asp Gln Gln Arg Leu Ile Phe Ala Gly Lys
35 40 45

Gln Leu Glu Asp Gly Arg Thr Leu Ala Asp Tyr Asn Ile Gln Lys Glu
50 55 60

Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly
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1 5 10

cgagct 53

<210> 4
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1 5 10

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<220>
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<222> ()..)
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<211> 33
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<220>
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<210> 8
<211> 18
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<210> 9
<211> 29
<212> DNA
<213> Plasmid pBIubi

<220>
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<210> 11
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<220>
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<223> Joining region of fusion of promoter and gene.

<400> 13
tctagaacta gtggatccct ggcatgcag 29

<210> 14
<211> 35
<212> DNA
<213> Plasmid pBIubi

<220>
<221> misc_feature
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<223> Joining region with multicloning sequence between
fusion of gene and terminator.

<400> 14
ggaggcctgt cgactcgagc ccgggtaccg agctc 35

WHAT IS CLAIMED IS:

1. A method for enhancing production of a desired protein in a plant cell or a plant which comprises inserting a first nucleic acid upstream of a second nucleic acid to form a fused nucleic acid wherein said first nucleic acid encodes a ubiquitin monomer and wherein said second nucleic acid encodes said desired protein and further wherein said fused nucleic acid encodes a fusion protein and further wherein expression of said fusion protein is not under the control of a ubiquitin promoter.

2. The method of claim 1 wherein said ubiquitin monomer consists of SEQ ID NO:2.

3. The method of claim 1 wherein the carboxy terminus of said ubiquitin forms a peptide linkage with the amino terminus of said desired protein.

4. The method of claim 1 wherein said first nucleic acid comprises bases 3-230 of SEQ ID NO:1.

5. The method of claim 1 wherein said fused nucleic acid is under the control of a 35S promoter.

6. A method for enhancing production of a desired protein as part of a fusion protein in a plant cell or a plant which comprises inserting a first nucleic acid upstream of a second nucleic acid to form a fused nucleic acid wherein said first nucleic acid encodes a protein of SEQ ID NO:4 and wherein said second nucleic acid encodes said desired protein and further wherein said fused nucleic acid encodes said fusion protein.

7. The method of claim 6 wherein the carboxy terminus of said protein of SEQ ID NO:4 forms a peptide linkage with the amino terminus of said desired protein.

8. The method of claim 6 wherein said first nucleic acid comprises bases 6-47 of SEQ ID NO:3.

9. The method of claim 6 wherein said fused nucleic acid is under the control of a 35S promoter.

10. A nucleic acid vector capable of transforming a plant cell wherein said vector comprises nucleic acid which encodes a fusion protein wherein said fusion protein comprises a ubiquitin monomer linked to a protein of interest and further wherein expression of said fusion protein is not under the control of a ubiquitin promoter.

11. The vector of claim 10 wherein said ubiquitin consists of SEQ ID NO:2.

12. The vector of claim 10 wherein said ubiquitin is linked in a peptide linkage at its carboxy terminus to the amino terminus of said protein of interest.

13. The vector of claim 10 wherein said nucleic acid is under the control of a 35S promoter.

14. The vector of claim 10 wherein said vector comprises bases 3-230 of SEQ ID NO:1.

15. A nucleic acid vector capable of transforming a plant cell wherein said vector comprises a nucleic acid which encodes a fusion protein wherein said fusion protein comprises a protein of SEQ ID NO:4 linked to a protein of interest.

16. The vector of claim 15 wherein said protein of SEQ ID NO:4 is linked in a peptide linkage at its carboxy terminus to the amino terminus of said protein of interest.

17. The vector of claim 15 wherein said nucleic acid is under the control of a 35S promoter.

18. The vector of claim 15 wherein said vector comprises bases 6-47 of SEQ ID NO:3.

19. A plant cell or a plant comprising the vector of claim 10.

20. A plant cell or a plant comprising the vector of claim 15.

21. A nucleic acid comprising SEQ ID NO:1.

22. A nucleic acid consisting of SEQ ID NO:1.

23. A nucleic acid comprising SEQ ID NO:3.

24. A nucleic acid consisting of SEQ ID NO:3.

25. A protein comprising SEQ ID NO:2.

26. A protein consisting of SEQ ID NO:2.

27. A protein consisting of SEQ ID NO:4.

28. A fusion protein wherein said fusion protein comprises a ubiquitin monomer at the amino terminus of said fusion protein and wherein said fusion protein comprises a second protein at its carboxy terminus.

29. The fusion protein of claim 28 wherein said ubiquitin monomer consists of SEQ ID NO:2.

30. The fusion protein of claim 28 wherein the carboxy terminus of said ubiquitin monomer forms a peptide linkage with the amino terminus of said second protein.

31. A fusion protein wherein said fusion protein comprises a protein of SEQ ID NO:4 at the amino terminus of said fusion protein and wherein said fusion protein comprises a second protein at its carboxy terminus.

32. The fusion protein of claim 31 wherein the carboxy terminus of said protein of SEQ ID NO:4 forms a peptide linkage with the amino terminus of said second protein.